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Cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol Import into Mitochondria through Mitochondrial Membrane Contact Sites in Permeabilized Rat Liver Hepatocytes

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The location of cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol import into mitochondria was examined in permeabilized rat liver hepatocytes. The model utilized the ability of intact mitochondria to freely import [^{14}C]glycerol-3-phosphate. Import of cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol was measured by the synthesis of phosphatidyl[^{14}C]glycerol. Phosphatidyl [^{14}C]glycerol was synthesized in a time- and concentration-dependent manner in the presence of cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol. The presence of ATP in the incubations stimulated phosphatidyl[^{14}C]-glycerol formation. The presence of adriamycin, at concentrations that block import of proteins into mitochondria, inhibited the formation of phosphatidyl [^{14}C]glycerol. In addition, adriamycin inhibited *in vitro* biosynthesis of phosphatidyl[^{14}C]glycerol in mitochondrial but not microsomal fractions. 1,4-Dinitrophenol, which may decrease the number of mitochondrial inner and outer membrane contact sites, inhibited formation of phosphatidyl[^{14}C]glycerol in permeabilized hepatocytes. The results demonstrate that cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol may be imported into mitochondria through inner and outer mitochondrial membrane contact sites in hepatocytes.

The movement of lipids and lipid precursors from their sites of synthesis to other organelles is an obligatory event for the biogenesis of biological membranes. Several mechanisms for mammalian lipid transport have been proposed including solubility and diffusion, soluble protein carriers, vesicle-mediated transport, target organelle-mediated modification of transported lipid and donor-target organelle fusion [for review see 1]. Mammalian mitochondria contain the enzymatic machinery for PG[1] biosynthesis [2-5] but must obtain several other phospholipids from the endoplasmic reticulum. CDP-DG, a precursor for PG biosynthesis, is synthesized by PA:CTP cytidylyltransferase [2]. In rat liver, the majority of PA:CTP cytidylyltransferase activity is located in the endoplasmic reticulum [3,4].

Kennedy and co-workers [2] demonstrated that PG could be synthesized in rat and chicken liver mitochondria in the presence of CDP-DG. Several other laboratories have demonstrated that the mitochondrial fractions of various tissues and cells had the capacity to promote PG biosynthesis [5-8]. In all of those studies biosynthesis of PG was exhibited only in the presence of exogenously added CDP-DG. The location of this liponucleotide import into the mitochondria was unknown. It was previously suggested that molecules such as free fatty acids, PA and liponucleotides may freely diffuse among organelles because of their appreciable solubility and polarity [for review see 9]. In this paper, we have solved one of the problems of measuring import of CDP-DG into the mitochondria via the use of permeabilized rat liver hepatocytes and exploitation of the rapid movement of G3P into mitochondria [10]. Using this approach both the synthesis of PG in the endoplasmic reticulum and the import of CDP-DG into mitochondria may be measured by the synthesis of [^{14}C]PG from [^{14}C]G3P. We provide strong evidence that extra-mitochondrial CDP-DG is imported into mitochondria through mitochondrial inner and outer membrane contact sites.

MATERIALS AND METHODS

[^{14}C]G3P was obtained from Amersham, International. Thin layer plates were obtained from Baxter CanLab, Winnipeg, Manitoba. Ecolite Scintillant was obtained from ICN Biochemicals, Costa Mesa, CA, U.S.A. Fetal bovine serum and Dulbecco's modified Eagle's medium were obtained from GIBCO. Dipalmitoyl CDP-DG was obtained

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Abbreviations: PG, phosphatidylglycerol; PA, phosphatidic acid; CDP-DG, cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol; DNP, 1,4-dinitrophenol; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PS, phosphatidylserine; G3P, glycerol-3-phosphate; ATP, adenosine-5′-triphosphate; CTP, cytidine-5′-triphosphate.

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from Serdary Research Laboratories, Englewood Cliffs, NJ, U.S.A. Adriamycin (Sigma D1515) was a generous gift of Dr. Leigh Murphy, Department of Biochemistry and Molecular Biology, University of Manitoba. All other biochemicals were of analytical grade and obtained from either Sigma Chemical Co., St. Louis, MO. U.S.A., or Fisher Scientific, Winnipeg, Manitoba, Canada. Male Sprague-Dawley rats (150-200 g) were used throughout the study. Rats had access to tap water and fed *ad libitum* in a temperature- and light controlled room. Treatment of animals conformed to the guidelines of the Canadian Council on Animal Care.

Isolation, incubation and permeabilization of hepatocytes. Hepatocytes were isolated by the collagen perfusion technique [11] except that medium containing 10% fetal bovine serum was used. Cells were plated at a density of 3-4 $\times$ 10^6 cells/60 mm diameter dish. The cells were incubated over night. The next morning Trypan blue was excluded by greater than 98% of isolated cells adhering to the dish. The medium was replaced with 2 ml of fresh medium minus serum and cells prepared for permeabilization 2 h later. Isolated intact hepatocytes were permeabilized by a modification of the method described by Voelker [12]. The dishes (3-4 $\times$ 10^6 cells/dish) were washed twice with 5 ml of Buffer A (0.14 M KCl, 10 mM NaCl, 2.5 mM $MgCl_2$, 0.1 μM $CaCl_2$, 20 mM HEPES, pH 7.6) at 37°C. Hepatocytes were permeabilized by incubation for 5 min with 5 ml of buffer A containing 50 μg/ml saponin at 37°C. The incubation medium was removed and the dishes bathed with 5 ml of ice cold buffer A. The cells (from 10-30 dishes) were harvested into 50 ml conical plastic centrifuge tubes and centrifuged at 300 $\times$ *g* for 5 min. The pellet was resuspended in buffer A to yield a cell density of 1.1-1.4 mg cell protein/0.25 ml. An aliquot of the cells were added to an equivalent volume of 0.16% Trypan blue dissolved in buffer A and staining of the cells examined under a light microscope. Greater than 96% of the cells were permeabilized as assessed by the inability of the cells to exclude Trypan blue. In two separate experiments mitochondria were estimated to be 81% and 86% intact as determined by the latency of cytochrome c oxidase activity compared with detergent treated cells as described [13]. Cell protein was determined by the method of Lowry *et al.* [14]. Bovine serum albumin was used as a protein standard.

Preparation of subcellular fractions and assay of in vitro PG biosynthesis. A 10% (by vol) rat liver homogenate was prepared in homogenizing buffer (0.145 M NaCl, 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) using 50 hand driven strokes of a tight fitting Dounce homogenizer. The homogenate was centrifuged at 1,000 $\times$ g for 5 min to cell unbroken cells and nuclei. The resulting supernatant was centrifuged at 10,000 $\times$ g for 15 min. The pellet was resuspended in homogenizing buffer and designated the mitochondrial fraction. The supernatant from this centrifugation was centrifuged at 105,000 $\times$ g for 60 min. The resulting pellet was resuspended in homogenizing buffer and designated the microsomal fraction. *In vitro* PG biosynthesis was measured by the conversion of [^{14}C]glycerol-3-phosphate and CDP-DG to the organic products PG phosphate and PG as described [5,6]. Incubations were performed in the absence or presence of 0.4 mM adriamycin. Product analysis revealed that greater than 75% of radioactivity was observed in PG and the remainder in PG phosphate.

Assay for CDP-DG import into mitochondria. Permeabilized cells (0.25 ml) were added to 13 $\times$ 100 mm screw cap incubation tubes. The incubation tubes contained 0.25 ml of buffer A containing the appropriate cocktail mixture (0.1 mM [^{14}C]G3P (0.44 μCi/tube) and CDP-DG (dipalmitoyl) in the absence or presence of 2.0 mM ATP, 5 mM phosphocreatine and 1 unit of creatine kinase or 0.1 mM CCCP or 0.1 mM DNP or 2 units apyrase or adriamycin. The mixture was incubated with light shaking at 37°C for up to 90 min. The reaction was terminated by the addition of 3 ml of chloroform:methanol (2:1, v/v). Phase separation was caused by the addition of 0.5 ml of 0.9% NaCl and resulting mixture vortexed, centrifuged and the aqueous phase removed. The organic phase was dried under nitrogen and resuspended in 50 μl of chloroform:methanol (2:1, v/v) and a 40 μl aliquot placed on a thin layer plate with PG standard. Plates were developed in a solvent system containing chloroform:hexane:methanol:acetic acid (50:30:10:5, by vol.). The plate was stained with iodine vapor and silica gel corresponding to PG standard was removed and placed in 7 ml scintillation vials. 5 ml of scintillant was added and the radioactivity determined by liquid scintillation spectrometry. Data was expressed as dpm/mg cell protein. In some experiments, the identification of radioactive PG was confirmed by two-dimensional thin-layer chromatography as described [5,6]. Students t-test was used for the determination of significance. The level of significance was defined as $P<0.05$.

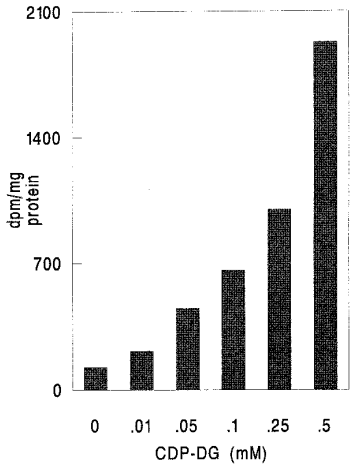

FIG. 1. The CDP-DG concentration-dependent synthesis of PG in permeabilized rat liver hepatocytes. Hepatocytes were permeabilized with saponin and incubated for 60 min with 0.1 mM [^{14}C]G3P in the absence or presence of various concentrations of CDP-DG and the radioactivity incorporated into PG was subsequently determined. Values represent the mean of two separate experiments each of which was performed in duplicate (background radioactivity was 20 dpm).

RESULTS

Since the majority of PG synthesis was reported to occur in the mitochondria of rat liver [3,15], the synthesis of [^{14}C]PG from [^{14}C]G3P and exogenous CDP-DG in permeabilized cells could be used as a measurement of CDP-DG import into the mitochondria. This should be an effective approach since [^{14}C]-G3P would freely enter mitochondria [10]. Rat liver hepatocytes were permeabilized with saponin and the cells were then harvested and incubated for 60 min with [^{14}C]G3P in the absence or presence of various concentrations of CDP-DG and the radioactivity incorporated into PG determined. The synthesis of [^{14}C]PG from [^{14}C]G3P was dependent on the concentration of CDP-DG (Fig. 1). Rat liver hepatocytes were permeabilized with saponin and the cells were then harvested and incubated for up to 90 min with [^{14}C]G3P in the absence or presence of CDP-DG and the radioactivity incorporated into PG determined. Radioactivity incorporated into PG in the absence of

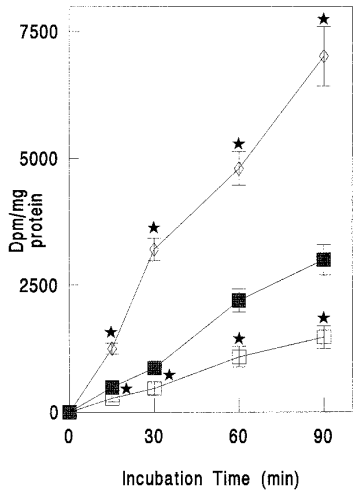

FIG. 2. The effect of adriamycin and ATP on PG biosynthesis in permeabilized rat liver hepatocytes. Hepatocytes were permeabilized with saponin and incubated for up to 90 min with 0.1 mM [^{14}C]G3P and 0.5 mM CDP-DG in the absence or presence of 0.4 mM adriamycin or 2.0 mM ATP and the radioactivity incorporated into PG subsequently determined. Values represent the mean ± standard deviation of three separate experiments each of which was performed in duplicate. Control, closed squares; 0.4 mM adriamycin, open squares; 2.0 mM ATP, open diamonds. The correlation coefficients for each line are: Control, 0.99858; adriamycin, 0.98411; ATP, 0.94676. ★$P<0.05$.

exogenous CDP-DG was approximately 80-90 dpm/mg protein (background 20 dpm) by 15 min of incubation and remained constant throughout the incubation period (data not shown). In the presence of CDP-DG, PG biosynthesis was synthesized with time for up to 90 min of incubation (Fig. 2). A 23-fold increase in PG synthesis was observed by 90 min of incubation with exogenous CDP-DG compared to incubations in which CDP-DG was absent. These data indicated that exogenous CDP-DG could be used for PG biosynthesis in permeabilized hepatocytes.

The import of PS into mitochondria of permeabilized Chinese hamster ovary cells is an ATP-driven process [12]. To determine if ATP stimulated import of CDP-DG into mitochondria, hepatocytes were permeabilized with saponin and incubated for up to 90 min with [^{14}C]G3P in the presence of CDP-DG in the absence or presence of ATP. As seen in Figure 2, ATP stimulated PG biosynthesis compared with incubations which contained CDP-DG alone. By 60 min if incubation a 2-fold ($P<0.05$) increase in PG biosynthesis was observed. We investigated if ATP associated with permeabilized cells (non-cytosolic pools such as the nucleus) could effect the synthesis of PG. Permeabilized hepatocytes were incubated with ATP and/or the ATP hydrolyzing enzyme apyrase or both and subsequently incubated with [^{14}C]G3P and CDP-DG. As seen in Table 1, when hepatocytes were incubated with apyrase and ATP, the ability of ATP to stimulate PG biosynthesis was abolished. In addition, the presence of apyrase alone did not effect PG biosynthesis compared to controls.

The antineoplastic drug, adriamycin, has been demonstrated to inhibit PS import into mitochondria in permeabilized Chinese hamster ovary cells [16]. Hepatocytes were permeabilized with saponin and incubated for up to 90 min with [^{14}C]G3P in the presence of CDP-DG in the absence or presence of adriamycin. The presence of adriamycin in these incubations inhibited the biosynthesis of PG from exogenous CDP-DG by approximately 50% at all times of incubation (Fig. 2). The presence of adriamycin did not affect the radioactivity incorporated into PG (80-90 dpm/mg protein) when CDP-DG was omitted from the incubations indicating that adriamycin itself did not affect [^{14}C]G3P movement into mitochondria. Previous studies had indicated that blockage of PS import into the mitochondria of permeabilized Chinese hamster ovary cells was dependent on the concentration of adriamycin [16]. Hepatocytes were permeabilized with saponin and incubated for 60 min with [^{14}C]G3P in the presence of CDP-DG in the absence or presence of various concentrations of adriamycin. The presence of adriamycin in these incubations inhibited the biosynthesis of [^{14}C]PG from [^{14}C]G3P and exogenous CDP-DG (Fig. 3). The inhibition of [^{14}C]PG biosynthesis was maximum between 0.4-0.6 mM adriamycin, concentrations which complex with almost all of the cardiolipin at the outer side of the inner mitochondrial membrane of rat liver [17]. The above findings are consistent with CDP-DG import into the mitochondria of permeabilized rat liver hepatocytes via mitochondrial inner and outer membrane contact sites.

DNP was shown to inhibit PS import into isolated rat liver mitochondria possibly by reducing the number

TABLE 1

The Effect of ATP, Apyrase, CCCP, and DNP on PG Biosynthesis in Permeabilized Rat Liver Hepatocytes

	Radioactivity incorporated into PG (dpm/mg protein)
Control	2073 ± 227
+ ATP	4763 ± 403*
+ ATP + apyrase	2360 ± 222
+ apyrase	2109 ± 197
+ CCCP	2182 ± 245
+ DNP	1454 ± 194*

Hepatocytes were permeabilized with saponin and incubated for 60 min with 0.1 mM [^{14}C]G3P and 0.5 mM CDP-DG in the absence or presence of 2.0 mM ATP or 2.0 mM ATP and 2 units apyrase or 2 units of apyrase or 0.1 mM CCCP or 0.1 mM DNP and the radioactivity incorporated into PG determined. Values represent the mean ± standard deviation of four separate experiments each of which was performed in duplicate. *$P < 0.05$.

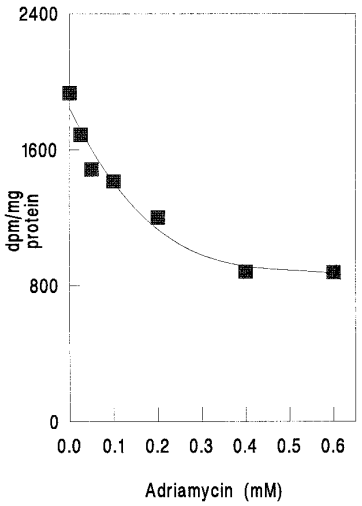

FIG. 3. The effect of various concentrations of adriamycin on PG biosynthesis in permeabilized rat liver hepatocytes. Hepatocytes were permeabilized and incubated for 60 min with 0.1 mM [^{14}C]G3P and 0.5 mM CDP-DG in the absence or presence of various concentrations of adriamycin and the radioactivity incorporated into PG was determined. Values represent the mean of two separate experiments each of which was performed in duplicate.

of mitochondrial inner and outer membrane contact sites [18]. Permeabilized hepatocytes were incubated for 60 min with [^{14}C]G3P and CDP-DG in the absence or presence DNP. The presence of DNP in the incubations inhibited PG biosynthesis by 30% ($P<0.05$) (Table 1). Since DNP is also a mitochondrial uncoupling agent, as a control, we determined if a functional proton electrochemical gradient was a prerequisite for CDP-DG import into mitochondria, permeabilized hepatocytes were incubated for 60 min with [^{14}C]G3P and CDP-DG in the absence or presence of CCCP. As seen in Table 1, CCCP was without effect indicating that a functional proton electrochemical gradient was not a prerequisite for CDP-DG import.

To determine if adriamycin affected CDP-DG import into mitochondria *in vitro*, PG biosynthesis from [^{14}C]-G3P and CDP-DG was examined in isolated crude rat liver mitochondrial fractions in the absence or presence of adriamycin. We examined the synthesis of PG in crude microsomal fractions since some PG is also synthesized in rat liver microsomes [3,15]. The presence of adriamycin inhibited PG biosynthesis by 34% ($P<0.05$) from 1.24 ± 0.11 to 0.82 ± 0.04 pmol/min · mg protein in mitochondrial fractions (average of four determinations). In contrast, PG biosynthesis in microsomal fractions was 0.36 ± 0.05 pmol/min · mg protein and unaltered by the presence of adriamycin indicating that adriamycin does not directly inhibit PG synthesis. Thus, in permeabilized cells adriamycin reduced the availability of CDP-DG for the PG biosynthetic machinery in the mitochondria. Taken together, all the above studies strongly support that the location of CDP-DG import into mitochondria occurs through mitochondrial inner and outer membrane contact sites.

DISCUSSION

In this study, the location of CDP-DG import into mitochondria was examined. The results of the present study suggest that extramitochondrial CDP-DG may be imported into mitochondria of permeabilized hepatocytes and this import occurs through mitochondrial inner and outer membrane contact zones. Extramitochondrial CDP-DG may contribute to the biosynthesis of PG in mitochondria *in vitro* [2,5-8]. Indeed, exogenous CDP-DG addition to isolated mitochondrial fractions is essential for the assay of PG phosphate synthase activity and the majority of this enzyme activity in rat liver mitochondria is localized on the inner membrane [19]. Previous studies had indicated that CDP-DG could be translocated from crude guinea pig liver microsomes to mitochondria [20,21]. However, mitochondrial fractions isolated by differential centrifugation contain a mixture of other membrane components and are not always completely intact. The use of the permeabilized cell model in our study eliminates the possibility of membrane mixing. In addition, the mitochondria in our study were essentially intact due to latency of cytochrome c oxidase. We observed both a concentration- and time-dependent synthesis of PG from extra-mitochondrial CDP-DG in permeabilized cells in our study indicating the ability of permeabilized cells to synthesize PG from exogenous CDP-DG. The adriamycin, ATP and DNP studies indicated that the mechanism of CDP-DG import into mitochondria was via movement through mitochondrial inner and outer membrane contact sites.

It is likely that some of the synthesis of PG in permeabilized cells incubated with [^{14}C]G3P and CDP-DG was due to synthesis at non-mitochondrial sites such as the endoplasmic reticulum. In fact, at concentrations of adriamycin that saturate inner and outer mitochondrial membrane contact sites significant (50% of control) synthesis of PG occurred. In addition, the *in vitro* studies with crude microsomes support PG synthesis in this local. However, import of CDP-DG into mitochondria likely accounted for at least 50% of PG biosynthesis in these experiments since; 1. adriamycin, known to inhibit protein and PS import into mitochondria [16,22], inhibited PG synthesis by 50% in permeabilized cells, 2. adriamycin inhibited PG biosynthesis in mitochondrial but not microsomal fractions *in vitro*, and 3. DNP inhibited PG synthesis in permeabilized cells consistent with this compounds ability to possibly decrease the number of contact sites [18]. Now that the location of CDP-DG import into mitochondria has been identified, studies on the mode of import (ie. diffusion or direct membrane contact between endoplasmic reticulum and mitochondria) may commence.

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